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Expression of wild type and mutant ELOVL4 in cell culture: subcellular localization and cell viability

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Purpose: *ELOVL4* is a member of the fatty acid elongase (*ELO*) family of genes. Mutations of this gene are responsible for autosomal dominant Stargardt-like macular degeneration. However, the specific role of ELOVL4 in photoreceptor cells and the mechanism by which mutations in *ELOVL4* causes macular degeneration are not known. In this study we examined the subcellular localization of wild type (wt) and mutant (mt) ELOVL4 EGFP fusion protein and the potential functional consequence of mtELOVL4 expression on cell viability.

Methods: Wt and mt ELOVL4 were expressed as EGFP fusion proteins in NIH 3T3 and HEK293 cells. Subcellular localizations of the fusion proteins were determined with a series of organelle-specific markers for endoplasmic reticulum (pDsRed2-ER), mitochondria (pDsRed2-Mito), peroxisomes (pDsRed2-Peroxi), and Golgi (BODIPY TR). Transfected cells were viewed using confocal and episcopic-fluorescence microscopy. Western blot analysis was performed to assess protein expression using an anti-GFP antibody. TUNEL staining was used to quantify apoptotic cell death.

Results: In cell transfection studies, wtELOVL4/EGFP fusion protein localized preferentially to the endoplasmic reticulum (ER) and was not found to be discernibly present in mitochondria, peroxisomes, or Golgi. In contrast, the truncated mutant fusion protein (which has no ER retention signal) appeared to be mislocalized to other compartments within transfected cells. Transfected cells expressing mtELOVL4/EGFP fusion protein exhibited induction of apoptotic cell death.

Conclusions: Unlike wtELOVL4/EGFP fusion protein, the mtELOVL4/EGFP fusion protein did not localize to the ER but rather appeared to be sequestered elsewhere in an aggregated pattern in the cytoplasm. The apoptosis induced by the mutant ELOVL4 fusion protein may be the mechanism whereby photoreceptor cells degenerate in Stargardt-like macular degeneration. Our study has provided an important in vitro model system for further assessment of ELOVL4 biochemical functions.

Macular degeneration is a heterogeneous group of disorders. The most prevalent form, age-related macular degeneration (AMD), is the leading cause of legal blindness in the elderly in developed countries. Stargardt-like macular dystrophy (STGD3, OMIM 600110) is an autosomal dominant form of juvenile macular degeneration characterized by decreased visual acuity, macular atrophy, and extensive flecks [1,2]. The disease shares some similarity with AMD including abnormal accumulation of lipofuscin in the retinal pigment epithelium (RPE) and degeneration of RPE and photoreceptors in the macula. The disease-causing gene *ELOVL4* encodes a protein with sequence and structural similarities to the ELO family of proteins. The ELO family of proteins is involved in the elongation of long chain fatty acids and is characterized by multiple putative membrane-spanning domains, a histidine cluster motif (HXXHH) involved in the enzymatic activity [3,4], and an ER retention signal [5]. Human ELOVL4 protein has 314 amino acids with a putative dilysine motifs (KXKXX) at the C-terminus thought to signal ER retention [6,7]. ELOVL4 and the ELO family members are thought to be a part of a complex enzymatic system, which participate in the catalysis of reduction reactions occurring during fatty acid elongation [8,9]. Elovl4 proteins are strongly conserved throughout vertebrate species [10,11].

Two *ELOVL4* mutations causing juvenile macular degeneration have been identified thus far. The first mutation is a 5 base-pair (bp) deletion starting at position 790 of the open reading frame (790_794delAACTT) [12]. This mutation results in a frame-shift and a premature stop codon. The resultant truncated protein contains seven aberrant amino acids and deletes a 51 amino acid fragment, including the dilysine ER retention signal at the C-terminus. The second mutation contains two 1 bp deletions, 789delT and 794delT [13], which produces a frame-shift nearly identical to the five base pair deletion.

To characterize the potential function of ELOVL4 and explore the functional consequence of the *ELOVL4* truncation, we investigated subcellular locations of normal and 5 bp deletion mutant ELOVL4 tagged with EGFP in tissue culture, and studied the effects of mutant *ELOVL4* on survival of transfected cells. Here, we show that the wild type (wt) ELOVL4 protein, as expected, localized to the ER compartment in transfected cells. In contrast, the truncated ELOVL4 was not retained in the ER, was found in an aggregated form in the cytoplasm of transfected cells, and eventually caused apoptotic

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cell death. Together, our results suggest that mislocalization of the mutant ELOVL4 protein, probably due to loss of the putative ER retention motif, and subsequent induction of apoptosis, may be the pathway leading to dominant Stargardt-like macular degeneration in the human retina.

METHODS

Materials: Media and reagents for cell culture and transfection were purchased from Gibco-BRL (Gibco, Grand Island, NY). Anti-EGFP monoclonal antibody and poly-L-lysine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Chamber slides were purchased from Nunc (Nalge Nunc International, Rochester, NY).

Generation of expression constructs: Wild type and 5 bp deletion mutant *ELOVL4* (wtELOVL4 and mt ELOVL4, respectively) cDNAs were cloned separately into a pEGFPC1 vector (Clontech, Palo Alto, CA). This vector utilizes a CMV promoter and expresses enhanced green florescent protein (EGFP) following transfection into mammalian cells. PCR was performed using one forward and two reverse primers 5'-CGG GGT ACC GCG ATG GGG CTC CTG GAC TC-3', 5'-CGGGATCCCG TTAATC TCC TTT TGC TTT TC-3', and 5'-CGGGATCCCG TTAGGC TCT TTG TAT GTC CGA-3' (containing *Kpn*1 and *Bam*H1 restriction sites) using wt and mutant *ELOVL4* cDNAs as templates. The resultant PCR products were digested with *Kpn*I and *Bam*HI, and cloned into the *Kpn*I and *Bam*HI sites of a pEGFPC1 vector in frame at the C-terminal end of EGFP (Figure 1) The recombinant plasmids containing EGFP-ELOVL4 fusion constructs were verified by direct DNA sequencing, amplified, and purified using a Qiagen plasmid isolation kit (Qiagen Inc., Valencia, CA).

Transfection studies and image acquisition: NIH3T3 and HEK293 were used for all transfection studies. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 i.u/ml of Penicillin, and 100 µg/ml of streptomycin. The recombinant plasmids were transfected into the cell lines using Lipofectamin Reagent 2000 (Gibco) according to the manufacturer's protocol. Cells were monitored for fluorescence between 7-36 h post transfection using episcopic-fluorescence microscopy.

NIH3T3 cells were seeded onto poly-L-lysine coated four well chamber glass slides at a confluence of 30% and transfected with recombinant plasmids containing wt or mt *ELOVL4*. Cotransfection with organelle specific markers pDsRed2ER, pDsRed2Mito, and pDsRed2peroxi (Clontech) were performed with wt and mt ELOVL4 to determine the subcellular localization of ELOVL4. Transfected cells were incubated at 37 C for 24 h, washed twice with phosphate buffer saline (PBS, pH 7.5), fixed in methanol:acetone (50:50, V/V) for 5 min at -20 C, the chambers removed and glass slides mounted with Vectashield mounting media (Vector laboratories, Inc., Burlingame, CA) for microscopy. To assess progressive subcellular localization of wt and mt protein ELOVL4, transfected cells were observed at different time intervals. However, all data presented in this study was collected at approximately the same time point after transfection.

For Golgi colocalization study, transfected cells were stained with BODIPY TR, a Golgi marker, per manufacturer's protocol (Molecular Probes, Inc., Eugene, OR)

Images of green fluorescence were collected using an Olympus IX70 confocal laser scanning microscope using 488 nm excitation source and 505-550 nm band pass barrier filter. Red fluorescence (DsRed2) markers for ER, mitochondria, and peroxisomes were examined using 568 nm excitation light from the He-Ne laser, a 575 nm dichroic mirror, and a 580-625 nm filter. The cells were illuminated only during image acquisition (3.7 s/frame for EGFP and DsRed2) and images were collected (in a section of 0.5 µm and pin hole size of 2 µm) where images were compared for co-localization analyses.

Electrophoresis and immunoblotting: To analyze the expression of wt and mt ELOVL4, transfected cells were grown

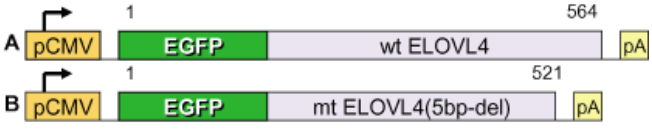

Figure 1. Recombinant constructs. Schematic diagram of recombinant constructs with position of amino acid depicted in numbers. **A**: EGFP and wild type ELOVL4 (wtELOVL4) fusion protein. **B**: EGFP and mutant ELOVL4 (mtELOVL4) fusion protein. Also labelled are the cytomegalovirus promoter (pCMV) and the Simian virus (SV) 40 polyadenylation signal (pA).

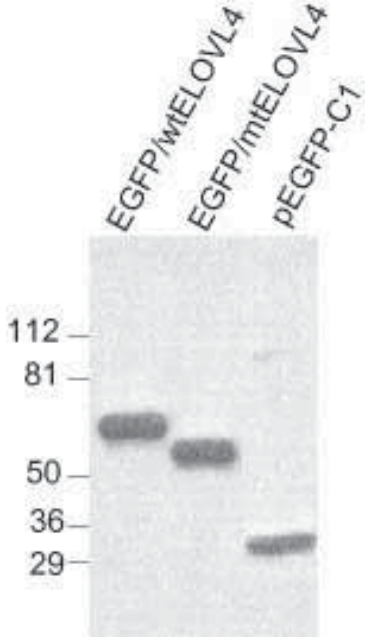

Figure 2. Western blot analysis of fusion proteins. Transfected HEK293 cells were allowed to express EGFP/ELOVL4 fusion proteins for 24 h, cells were harvested, followed by immunoblotting using anti-GFP monoclonal antibody. Lane 1 is EGFP/wtELOVL4. Lane 2 is EGFP/mtELOVL4. Lane 3 is EGFP alone as a vector control.

for 24 h, harvested from the plates, and briefly washed with PBS. Cells were lysed on ice for 20 min with a buffer containing 1% Triton X-100, 0.01% SDS, 0.05 M Tris-HCl, and 0.001 M EDTA (pH 7.5). The cell lysates were centrifuged at 4000 rpm for 5 min and supernatants used for electrophoresis.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [14]. Ten µl of sample (about 7 µg protein) was loaded onto a 9% polyacrylamide gel and electrophoresed at 110 V for 1 h. The resolved proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), and blocked for 2 h at room temperature with 5% skim milk in Tris buffered saline containing 0.05% Tween 20 (TTBS). The membrane was incubated for 2 h with monoclonal antiGFP antibody diluted 1:2000 in 5% milk containing TTBS, and then probed with peroxidase-conjugated antimouse antibody (1:4000 dilution in TTBS, Amersham Biosciences, NJ) for 1 h and developed with an ECL (Amersham Bioscience) detection kit according to the manufacturers' protocol.

Apoptosis assay in transfected cultured cells: An in situ apoptotic cell death detection kit TMR red (Roche Applied Science, Indianapolis, IN) based on a TUNEL assay, was used to detect apoptotic cells in cultures expressing wt or mt ELOVL4. The TUNEL assay was performed on transfected cells after 20 h incubation, as per manufacturer's protocol. Apoptotic cells were visualized using episcopic-fluorescence microscopy at 20x and 40x magnification. Transfection positive cells were scored by green fluorescence and tunnel positive cells scored by red nuclear staining using 20x magnification. Data were collected from 3 separate sets of transfection

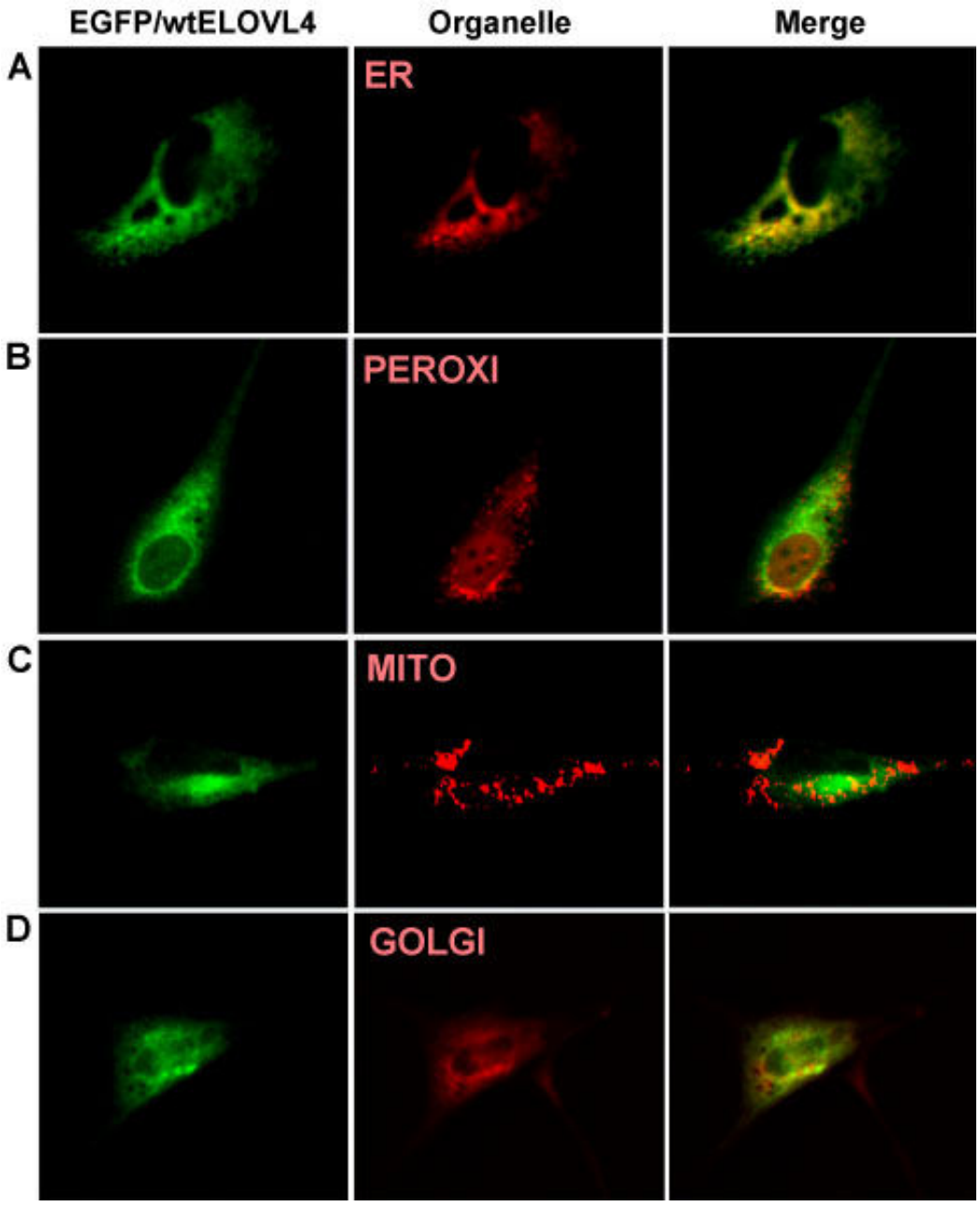

Figure 3. Intracellular localication of EGFP/wt ELOVL4 fusion protein. Localization of wtELOVL4-EGFP fusion protein with different organelle specific markers in NIH3T3 cells. The cells were imaged using confocal microscopy 24 h following co-transfection. Green images represent the expression of ELOVL4 and red images represent different organelles. Merged color image: superimposed image of green and red. **A**: Co-transfected cell with EGFP/wtELOVL4 and pDsRed2-ER (specific for Endoplasmic Reticulum). **B**: Co-transfected cell with EGFP/wtELOVL4 and pDsRed2-Peroxi (specific for peroxisomes). **C**: co-transfected cell with EGFP/wtELOVL4 and PDsRed2-Mito (specific for mitochondria). **D**: Cells were transfected with EGFP/wtELOVL4 and stained with Golgi specific marker BODYPY (red).

experiments (duplicate wells). Results are presented as percent apoptotic cells per 550 EGFP-ELOVL4 fusion protein positive cells.

RESULTS

Characterization of EGFP-ELOVL4 fusion proteins: In this study we expressed wt and mt ELOVL4 as EGFP fusion proteins to facilitate direct visualization of subcellular localization (Figure 1). Western blot analysis confirmed the synthesis of EGFP-ELOVL4 fusion proteins; single bands were visualized for each construct at approximately 61 kDa for wt ELOVL4 and approximately 56 kDa for the EGFP-ELOVL4 truncated mutant (Figure 2). Culture medium was also examined by western blot analyses; no detectable protein was found (data not shown).

Subcellular localization of ELOVL4: Episcopic-fluorescence and confocal microscopy were used to determine the subcellular localization of wt and mt ELOVL4 in transfected

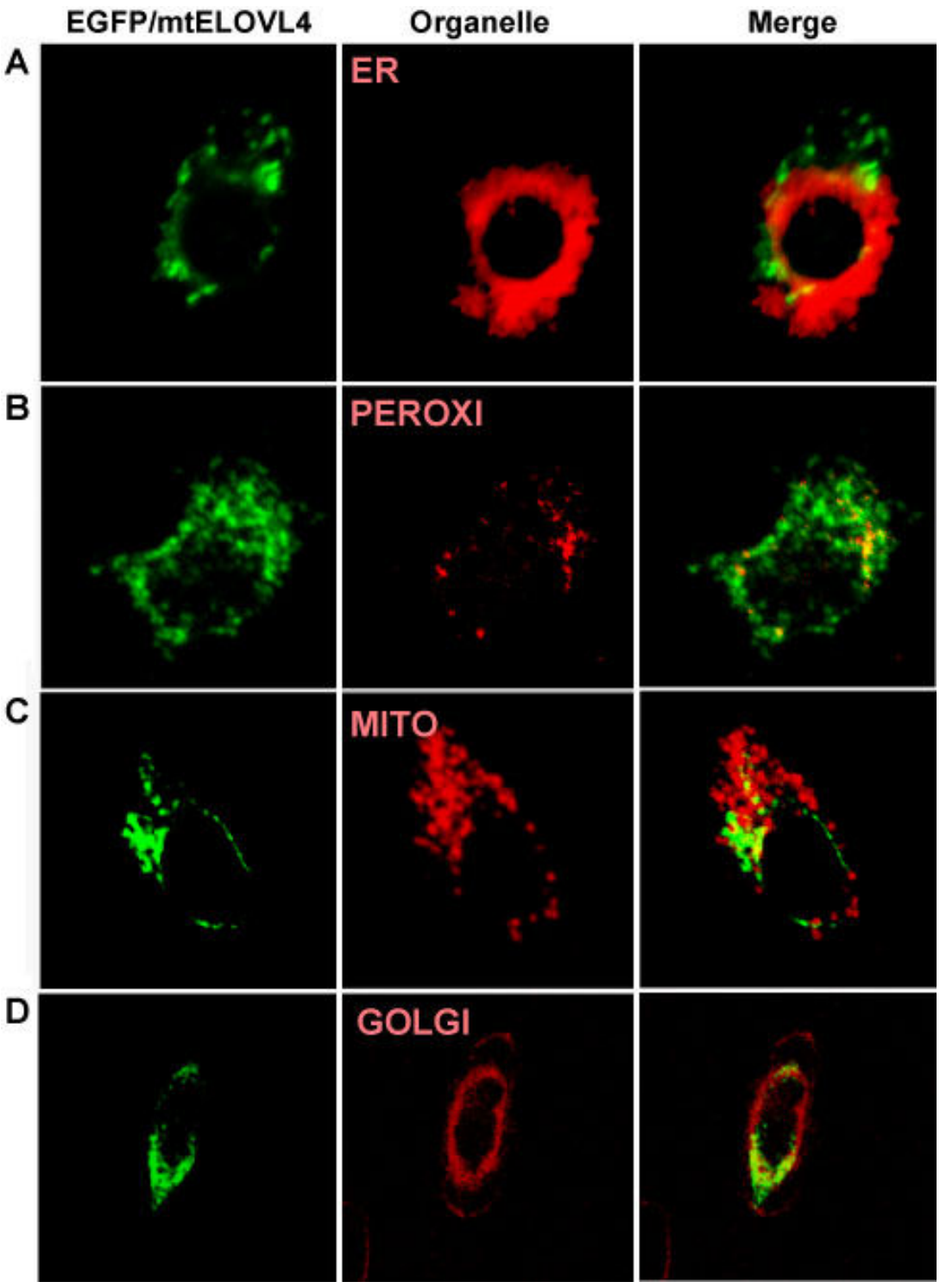

Figure 4. Intracellular localization of EGFP/mutant ELOVL4 protein. Localization of EGFP/mtELOVL4 with different organelle-specific markers in NIH3T3 cells. Transfection and imaging were performed in the same manner described in Figure 3. **A**: Co-transfected cell with EGFP/mtELOVL4 and pDsRed2-ER (specific for ER). **B**: Co-transfected cell with EGFP/mtELOVL4 and pDsRed2-Peroxi (specific for peroxisomes). **C**: Co-transfected cell with EGFP/mtELOVL4 and pDsRed2-Mito (specific for mitochondria). **D**: Cells were transfected with EGFP/mtELOVL4 and stained with Golgi specific marker BODYPY (red).

cells Wild type ELOVL4 localized preferentially to the ER compartment (Figure 3A), but showed no evidence of co-localization in peroxisomes when cotransfected with DsRed peroxisomes (Peroxi; Figure 3B), nor mitochondria (Figure 3C) when cotransfected with DsRed mitochondria (Mito) markers. There was also very little Golgi co-localization of wt ELOVL4 when transfected cells were co-stained with BODIPY TR, a Golgi marker (Figure 3D). In contrast, mtELOVL4 showed markedly different subcellular localization pattern in the transfected cells. It did not localize to the ER (Figure 4A), peroxisomes (Figure 4B), mitochondria (Figure 4C), or Golgi (Figure 4D). Instead, the pattern of its subcellular distribution is consistent with an aggregated form in the cytoplasm. As a control, EGFP when expressed alone, localized to the cytoplasm as reported previously (data not shown) [15].

Cytotoxicity of mtELOVL4/EGFP fusion protein: We found that a significant number of transfected cells expressing mutant (mt) ELOVL4/EGFP fusion protein died in culture. This observation prompted us to investigate whether the mutant induces cells to undergo programmed cell death. An in situ cell death assay (TUNEL) for apoptosis was performed. Transfected cell populations expressing mtELOVL4 showed a marked increase in apoptotic cell death as evidenced by positive TUNEL staining in comparison to cells expressing wtELOVL4. Specifically, a nine-fold (about 23% of 560 total number of cells) induction in apoptotic cell death was observed when the cells were transfected with mtELOVL4-EGFP fusion plasmid (Figure 5). In contrast, cells transfected with wtELOVL4-EGFP fusion plasmid showed a level of apoptosis comparable to that of EGFP expression vector (Figure 5). Therefore, overexpression of wtELOVL4-EGFP fusion protein does not induce apoptosis.

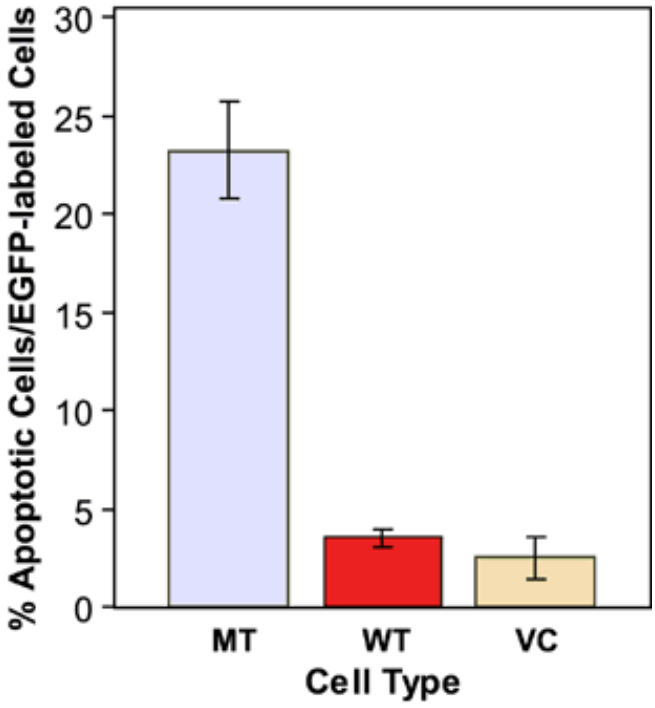

Figure 5. Apoptosis induced by fusion protein expression. TUNEL assay of transfected HEK293 cells expressing EGFP/wtELOVL4 (WT) or EGFP/mtELOVL4 (MT) fusion proteins. The TUNEL assay of vector control (VC) is co-plotted. Assays were performed after 20 h of transfection. After a TUNEL reaction, cells were observed using episcopic-fluorescence microscope. The green channel was used to detect fusion protein expression and red channel was used to detect apoptotic nuclear staining. Results were plotted as percent apoptotic cells per equivalent number of transfected cells.

DISCUSSION

Stargardt-like macular dystrophy (STGD3) is an autosomal dominant form of juvenile macular degeneration and is characterized by bilateral atrophic changes in the macula, degeneration of the underlying RPE, and the presence of prominent flecks in the posterior pole[1,12,16]. The disease-causing gene ELOVL4 encodes a protein with similarities to a family of proteins involved in the elongation of long chain fatty acids. Since normal ELOVL4 functions in photoreceptors, and the mechanism whereby mutations in ELOVL4 lead to macular degeneration are unknown, we investigated subcellular distribution of normal and mutant ELOVL4 and effects of mutant protein expression on cell viability.

Given the sequence similarity of ELOVL4 to a family of proteins functioning in elongation of long chain fatty acids, including dilysine ER retention signal, one would expect that normal ELOVL4 localizes to ER, the site of protein synthesis and fatty acid elongation. Indeed, our results of NIH3T3 cell transfection studies indicated ER localization for normal ELOVL4. To avoid bias of NIH3T3 cell lines, we also found ER localization of ELOVL4-EGFP fusion protein in two other cell lines, HEK293 and COS7 (data not shown). Although there is a small possibility that ER localization of EGFP-ELOVL4 is an artifact of the fusion protein, we believe that the ER localization represents authentic location of ELOVL4 native protein for the following reasons: First, our control transfection experiments showed that EGFP when expressed alone localized to the cytoplasm (data not shown); second, the EGFP/mtELOVL4 lacking 51 C-terminal amino acids including a dilysine ER retention signal, did not localize to the ER. Further experiments using ELOVL4-specific antibodies will verify the above findings.

Unlike wtELOVL4-EGFP fusion protein, the mutant does not localize to the ER but rather appears to be sequestered elsewhere, showing dense fluorescent-positive aggregates. Utilizing the available organelle markers on hand, we found that the mtELOVL4-EGFP fusion protein does not reside within peroxisomes, Golgi, or mitochondria but rather some alternative unidentified compartment. Based on our microscopic studies, it is possible that the truncation of ELOVL4 renders the polypeptide prone to form high molecular weight oligomeric species in the cytoplasm of transfected cells, accumulating in an aggregated form. Future studies with additional cell markers and/or electron microscopy will elucidate more precisely the subcompartmental localization of the mutant protein.

We demonstrated that mislocalized mtELOVL4-EGFP fusion protein induces apoptotic cell death in HEK293 cells.

We also observied similar findings in NIH3T3 and COS7 cell lines (data not shown). There is a nine-fold increase in TUNEL reactivity in transfected HEK293 cell populations expressing mtELOVL4-EGFP fusion protein, relative to that expressing wtELOVL4-EGFP fusion protein. The above results support a model that dominant negative effects rather then haploid insufficiency of ELOVL4 is a basis for retinal photoreceptor degeneration in Stargardt-like macular dystrophy. Apoptosis induced by mutant ELOVL4 could be a general mechanism rather than a photoreceptor-specific one, since apoptosis was observed in non-photoreceptor cells.

Although the primary sequence of ELOVL4 shows strong sequence similarity to a family of conserved proteins involved in elongation of long chain fatty acids, its putative enzymatic function has not been established. Our study has provided an important in vitro model system for further assessment of ELOVL4 biochemical functions.

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